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Short communication

Optimized extraction of a lectin from *Crataeva tapia* bark using AOT in isooctane reversed micelles

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ABSTRACT

Crataeva tapia bark lectin was extracted from a crude extract into a reversed micelle phase of the anionic surfactant AOT in isooctane and back-extracted, to a final aqueous phase by addition of butanol. The effects of pH, ionic strength and surfactant concentration on the protein transfer process from the aqueous to the organic phase were characterized, being the best results obtained after 5 min of contact, under agitation, between the two phases, at pH 5.5 (10 mM citrate-phosphate buffer), 30 mM NaCl, and 5 mM AOT. Recovery to a new aqueous phase was performed with 5 min of contact, under agitation, 10 mM citrate-phosphate buffer at pH 5.5, 500 mM KCl and 5% of butanol. The overall yield obtained for the process was 80% for lectin activity and 56% for protein recovery. The efficiency of the process was confirmed by SDS-PAGE analysis.

1. Introduction

Lectins occur ubiquitously in nature and are important in medicine and technology. Most lectins are basically non-enzymic in action and non-immune in origin. They may bind to a carbohydrate moiety that is in solution or to one that is a part of protein/particulate body. They can agglutinate cells and/or precipitates glycoconjugates [1]. Plant seeds and bark provide a major source from which lectins are isolated. Some plant lectins appear to have pharmacological activity [2]. For example, in the northeast of Brazil *Crataeva tapia* bark infusions have been used in popular medicine as hypoglycemic agent.

Protein extraction from aqueous solution by reverse micelles is a process that utilizes basic techniques of chemical engineering such as classical liquid–liquid extraction, and thus has the potential for industrial application [3]. This process can be used to separate biological products such as, proteins, which have been solubilized in organic solvents using surfactants, without affecting their functional properties [4]. The process can be performed by a forward extraction of the target protein or contaminants, from an aqueous solution to a reversed micellar organic phase, followed by back-extraction, during which the biomolecules are released from micelles and transferred to a new aqueous phase [5], recent examples being the extraction of a lysozyme and ovalbumin [6], chitanases [7], α-amylase [8] and nattokinase [9].

In this work, the extraction and purification of a new thermostable plant lectin from *C. tapia* bark was studied using a reversed micelle system of the anionic surfactant sodium di(2-ethylhexyl)sulfosuccinate (AOT) in isooctane. Process optimization was performed by addition of butanol and manipulation of pH, ionic strength and surfactant concentration.

2. Materials and methods

2.1. Chemicals

Sodium di(2-ethylhexyl)sulfosuccinate was obtained from Sigma (USA); isooctane, butanol and glutaraldehyde were obtained from Merck (Germany); bicinchoninic acid (BCA) was obtained from Pierce. All other chemicals were of analytical grade.

2.2. Preparation of extract

C. tapia bark was collected in the region of Recife city (Pernambuco, Brazil) and the extract was obtained by pulverizing dried bark [10% (w/v) in 150 mM NaCl] followed by agitation overnight at 4 °C. Afterwards, the extract was filtered through

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a gauze and the filtrate centrifuged at 4000 × *g* for 15 min. The supernatant was termed crude extract (CE).

2.3. Extraction and back-extraction of protein with reversed micelles

The reversed micellar system was constituted using the anionic surfactant, sodium di(2-ethylhexyl)sulfosuccinate in isooctane. Extraction and back-extraction procedures were performed as follows: (1) to buffered lectin preparations (3 ml) at different pH (10 mM citrate-phosphate, pH 3.0–6.0, 10 mM sodium-phosphate, pH 7.0, 10 mM Tris–HCl, pH 8.0, 10 mM carbonate–bicarbonate, pH 9.0–10.0 and 10 mM glycine–NaOH, pH 11.0–12.0) containing 30 mM NaCl, an equal volume (3 ml) of micellar phase (AOT in isooctane at a concentration range of 0.125–100 mM) was added and both phases were stirred for 5 min for protein extraction. The mixture was then centrifuged for 5 min at 3000 × *g*, for phase separation. (2) After extraction, the separated micellar phase (2 ml), containing solubilized protein, was added to an equivalent volume of buffered aqueous solution at different pH (10 mM citrate-phosphate, pH 5.0–6.0 and 10 mM sodium-phosphate, pH 7.0) containing 50–1000 mM KCl and 5% (v/v) of butanol was added to the system. The mixture was stirred for 5 min, centrifuged for 5 min at 3000 × *g* for phase separation and the lectin recovered to the new aqueous phase. Agitation speed (700 rpm), temperature (25 °C) and initial protein concentration (0.374 mg/ml) were kept constant in all experiments.

2.4. Protein assays

The protein content in the aqueous and organic phases was spectrophotometrically determined using the bicinchoninic acid according to Smith et al. [10], with bovine serum albumin as standard, at a range of 0–600 μg/ml.

2.5. Determination of the hemagglutinating activity

The determination of the hemagglutinating activity (HA) in the aqueous phase was performed in microtiter plates according to Correia and Coelho [11]. Lectin preparations (50 μl) were two-fold serially diluted with 0.15 M NaCl before addition of 50 μl suspension of rabbit erythrocytes treated with glutaraldehyde 2.5% (v/v). The HA was expressed as the log of the highest dilution exhibiting hemagglutination. The determination of the activity in the back-extraction aqueous phase was carried out after dialysis due to the interference of salt concentration used. The protein hemagglutinating activity was not measured in organic phase, due to interference of the organic solvent (micellar phase). The specific HA (SHA) was given by the ratio between HA and protein content (mg/ml). The purification factor (PF) as follows:

$$\mathrm{PF} = \frac{\text{SHA in new aqueous phase after back-extraction}}{\text{SHA in initial aqueous phase}} \quad (1)$$

2.6. Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed on 10% (w/v) gel according to Laemmli [12]. Polypeptide bands of lectin (150 μg of protein) and standards [bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; lysozyme, 14.3 kDa from Sigma (USA)] were stained with Coomassie Brilliant Blue. PAGE for native basic [7.5% (w/v) gel] and acidic [12% (w/v) gel] proteins were performed according to Reisfeld et al. [13] and Davis [14], respectively.

2.7. Gel filtration chromatography

Lectin extracted by reversed micelles was chromatographed on gel filtration on a Hiprep 16/60 Sephacryl S-300 column (16 mm × 60 cm)/Akta FPLC system (Amersham Pharmacia Biotech, Sweden) pre-equilibrated at 24 °C with 0.5 M NaCl. Samples (2.0 ml; 1 mg of protein) were injected and eluted with the same solution at a flow rate of 3.0 ml/min. The standards (Sigma, USA) similarly chromatographed were bovine serum albumin (66 kDa), fetuin (64 kDa), ovalbumin (45 kDa) and trypsin inhibitor type III-O chicken (28 kDa).

3. Results and discussion

3.1. Effect of pH on the extraction

The major determining factors on protein solubilization in reversed micellar systems are electrostatic interactions among biomolecules and charged surfactant heads as well as the aggregation properties of surfactant. pH influence on lectin extraction was evaluated for a 30 mM NaCl aqueous phase and 5 mM AOT/isooctane. The pH of the aqueous phase (CE) was varied between 3.0 and 12.0, using different buffer systems according to their pK_a value (Fig. 1). The results showed a high transfer of protein to the micellar phase within the pH range of 3.0–6.0, with a maximum of ca. 70% at pH 5.5. A similar situation has been reported for *Cratylia mollis* seed lectin [15], with a maximum extraction at pH 5.0. This behaviour suggests that pH 5.5 was more selective, probably due to favorable attractive electrostatic interactions between positively charged lectin molecules at pH 5.5 (lectin has a p*I* 9.5 as previously determined in our laboratory) and negatively charged surfactant heads. For smaller proteins, using anionic surfactants at pH conditions below the protein p*I* their transfer from aqueous to micellar solution is favoured [16]. As the pH of aqueous phase increased from 5.5 to 12, the extracted protein decreased ca. of 34% probably due to the proximity of the isoelectric point of the lectin. This phenomenon was observed for different proteins at distinct isoelectric points [17]. The protein hydrophobic patches exposed may interact with anionic surfactant hydrophobic tails minimizing extraction. Besides the protein charge, the density of surface charge is an important factor on protein solubilization. Therefore, the selected pH to be used in further experiments was 5.5.

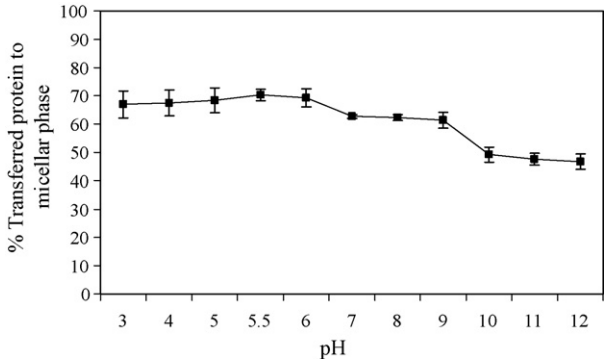

Fig. 1. Effect of pH on lectin extraction with 30 mM NaCl, 5 mM AOT in isooctane reversed micelles. Buffers: pH 3.0–6.0 (10 mM citrate-phosphate); pH 7.0 (10 mM sodium-phosphate); pH 8.0 (10 mM Tris–HCl); pH 9.0–10.0 (10 mM carbonate–sodium bicarbonate); pH 11.0–12.0 (10 mM glycine–NaOH). Each data point is an average of three experiments and the error bars show the standard deviation.

3.2. Effect of AOT concentration on the extraction

Protein solubilization is strongly dependent on the concentration of surfactant and on the size of the micelle relative to that of the protein. Before analysing the effect of AOT concentration on lectin purification the critical micellar concentration was determined as being 1 mM AOT, which is in agreement with results presented by Yan-ching and Ache [18]. For a AOT concentration of 0.125 mM, no phase separation occurred and for AOT concentrations of 0.25 and 0.50 mM an apparent phase separation (aqueous phase/micellar phase) was observed, but no protein was transferred to the micellar phase while on the other hand, 78.8 and 82.11% of protein formed a clearly visible precipitate at the interface, respectively. For AOT concentrations higher than the cmc (1–100 mM AOT), the influence of AOT concentration on lectin extraction under 30 mM of NaCl and pH 5.5, was evaluated (Fig. 2). The results showed that protein extraction was ca. 60% at AOT concentration between 1 and 3 mM, remaining constant at 70% for AOT concentrations between 5 and 50 mM with a 5% decrease for 100 mM AOT. It is well known that the increase in the amount of surfactant in organic phase leads to an increase of protein solubilization due to the increase of the amount of surfactant aggregation and/or the increase of size of reverse micelles [19]. Nevertheless, no significant differences were found on extraction over AOT concentration range of 5–50 mM. These results could be explained by the size of lectin associated to a strong electrostatic

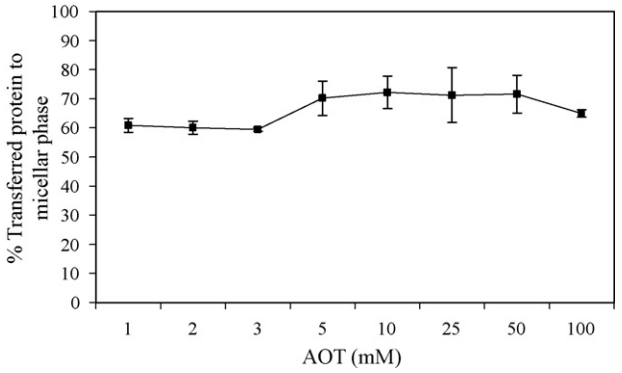

Fig. 2. Effect of AOT concentration on lectin extraction under 30 mM of NaCl and pH 5.5 conditions during forward transfer to micellar phase. Each data point is an average of three experiments and the error bars show the standard deviation.

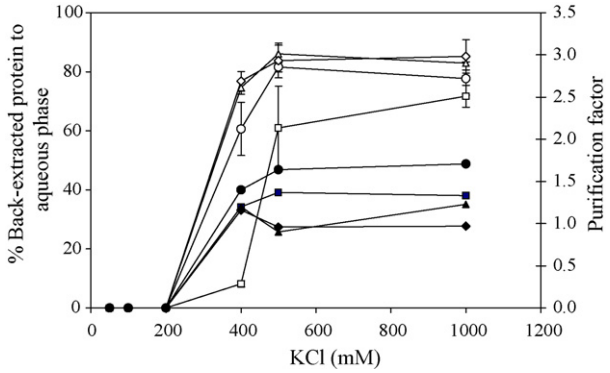

Fig. 3. Effect of pH and ionic strength on back-extraction at pH 5.5, with 5 mM AOT in isooctane reversed micelles to a new 10 mM buffered aqueous phase at pH 5.0 (□), 5.5 (○) and 6.0 (△) (with citrate-phosphate) and pH 7.0 (◇) (with sodium-phosphate) with addition of KCl concentrations of 50–1000 mM. Initial concentration of protein in micellar phase = 0.260 mg/ml and initial log HA = 1.5. Key: open symbols, % back-extracted protein to aqueous phase; closed symbols, purification factor. Each data point is an average of three experiments and the error bars show the standard deviation.

interaction between protein and surfactant due to the basic nature of the lectin. A similar situation has been reported for the extraction of a lipase from *Penicillium citrinum* across an AOT concentration range of 100–350 mM [20]. Taking in account these results, the 5 mM AOT concentration was chosen for the following experiments since it might be expected that a high surfactant concentration makes difficult the backward transfer of proteins into a second aqueous phase [4].

3.3. Effect of pH and ionic strength on the back-extraction

The back-extraction step was more difficult to accomplish, probably due either to the strong electrostatic interactions between positive lectin charge and negative charge of AOT or possibly the high affinity between the protein and the surfactant. A small amount of alcohol added to an organic solution can improve the back-extraction behaviour of proteins depending on the concentration and alcohol species used [21]. For example, the presence of butanol in the system may change the water properties inside reversed micelles [22] and may affect protein–micelle interactions in a fundamental manner [23]. For this reason 5% butanol was used during back-extraction. Fig. 2 shows that the amount of protein back-extracted to the new aqueous phase increased with the increase of pH value and KCl concentration with a maximum recovery (85%) at pH 7.0 containing 500 mM KCl. A further increase in the KCl concentration did not result in a higher content of back-extracted protein or a higher purification factor. This electrostatic screening effect may also be responsible for the decrease of the surfactant head group repulsions, leading to smaller reversed micelles [4], which in turn, could be a factor responsible for the higher back-extraction of proteins to the aqueous solution. However, we found that KCl concentrations higher than 500 mM did not increase the recovery of the protein. This may well be due to the fact that the electrostatic effect reached a maximum at 500 mM KCl. While the higher back-extraction (85%) was found at pH 7.0, a better purification factor (1.7) was obtained at pH 5.5 (Fig. 3) with 500 mM KCl. These results suggest that the increased percentage of protein transferred into a new aqueous phase when pH was increased from 5.5 to 7.0 was not due to higher lectin content but to other contaminant proteins. Similar purification factors of protein, 1.8 and 1.5, using micellar systems have been reported for an extracellular alkaline protease from fermentation broth [22] and for a xylanase from fermentation broth [24], respectively. The presence of butanol in the system permitted the back-extraction of lectin from a reversed micellar phase to an aqueous phase, as a consequence of a structural factor change of the micelles, corroborated by Liu et al. [9], and Lee et al.

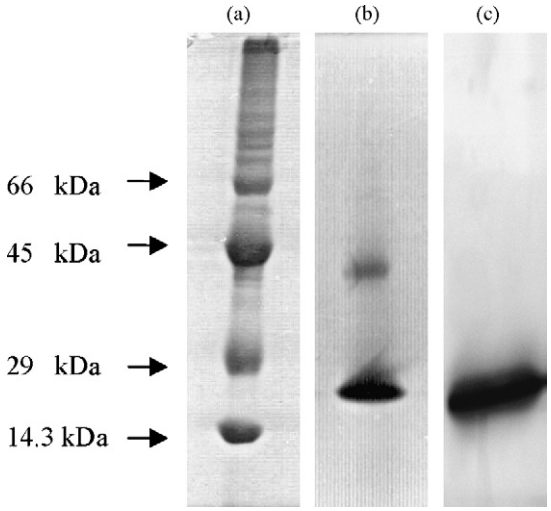

Fig. 4. PAGE of back-extracted sample at pH 5.5, containing 500 mM KCl with the addition of 5% (v/v) butanol at 25 °C. SDS-PAGE: (a) molecular weight markers and (b) lectin purified by reversed micelles; (c) PAGE for native basic protein: lectin purified by reversed micelles.

[21]. The optimal condition for direct extraction of lectin from crude extract led to a recovery of 56% with retention of, at least, 80% of the lectin HA.

3.4. Structural characterization of purified lectin by reversed micellar system

Electrophoresis was applied to the purified lectin as previously described. SDS-PAGE showed two polypeptide bands of molecular mass 21 and 40 kDa (Fig. 4a and b) while PAGE for native basic protein revealed a single lectin band (Fig. 4c) and no acidic protein band was detected. The evaluation of purified lectin by gel filtration chromatography (Fig. 5a) revealed two protein peaks corresponding to 40 and 29 kDa. When the crude extract was applied to the chromatography column (Fig. 5b) one main peak of 40 kDa and additional peaks of 26, 19 and 7.6 kDa were detected. At 29 kDa, a very small peak was also observed. The chromatographic profiles obtained indicate the efficiency of the reversed micellar system for lectin purification as none of the contaminants detected on the crude extract were observed in the purified

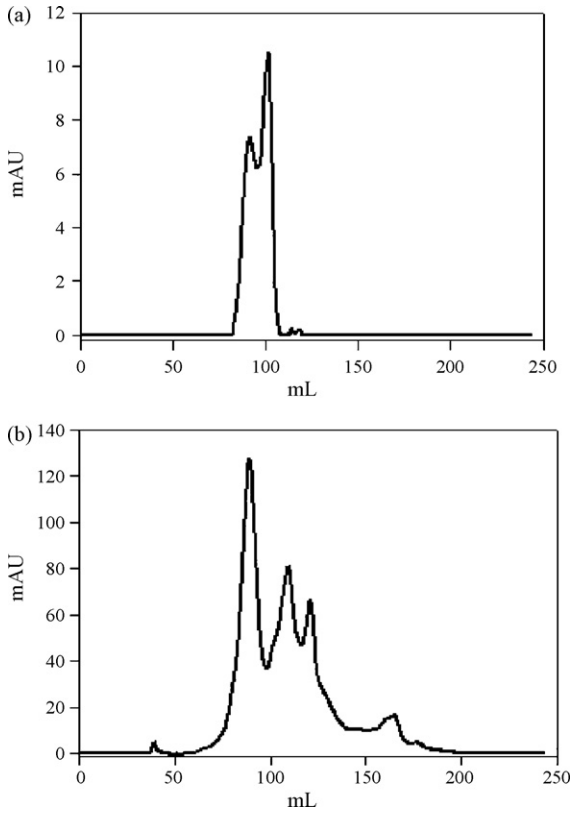

Fig. 5. Gel filtration chromatography on Sephacryl S-300: (a) purified lectin (2 ml; 0.3 mg) applied on column (16 mm × 60 cm) equilibrated with 500 mM NaCl and (b) crude extract (2 ml; 4 mg). Fractions (3 ml) were collected at 1.0 ml/min.

preparation. The two peaks detected by gel filtration chromatography under non-denaturing conditions for the purified lectin reflect the presence of aggregated and non-aggregated protein as already observed in SDS-PAGE (Fig. 4b). The obtention of a 29 kDa fraction by gel filtration chromatography, as compared to the 21 kDa fraction obtained by SDS, may be due to the incomplete unfolding of the non-aggregated lectin that occurs on the presence of SDS denaturing conditions.

4. Conclusion

The utilization of reverse micelles on lectin extraction and purification from a crude extract was successfully applied using an AOT/isooctane/water biphasic system with the addition of butanol on the back-extraction step and adjustment of pH, ionic strength and AOT concentration. Although the maximum recovery (85%) was found to be at pH 7.0 with 500 mM KCl, the highest purification factor (1.7) was found to be at the same pH level (5.5) found on the extraction step. Being so, the pH value of 5.5 was established as a compromise between the maximum activity (80%) and protein (56%) recovery. These results were obtained using a 10 mM citrate-phosphate buffer solution, pH 5.5 containing 30 mM NaCl; 5 mM AOT and 5 min agitation for the extraction step and a 10 mM citrate-phosphate buffer solution, pH 5.5 containing 500 mM KCl plus 5% of butanol and 5 min agitation for the back-extraction. This purification procedure allows for the obtention of a high purity lectin allowing for its further *in vivo* evaluation as a hypoglycemic agent.

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